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Abstract

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Characterization of the O-Polysaccharide Structure of Lipopolysaccharide from *Actinobacillus actinomycetemcomitans* Serotype b

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We previously reported that the serotype b antigen of *Actinobacillus actinomycetemcomitans* is a constituent of the polysaccharide region of lipopolysaccharide (LPS) and contains significant amounts of the neutral sugars rhamnose and fucose (M. Wilson and R. Schifferle, Infect. Immun. 59:1544–1551, 1991). In the present study, we determined the structure of the O antigen of *A. actinomycetemcomitans* Y4 (serotype b) LPS. Aqueous-phase LPS was obtained from a phenol-water extract of *A. actinomycetemcomitans* Y4. This material was found to react with rabbit polyclonal antiserum to serotype b but not with antisera specific for other *A. actinomycetemcomitans* serotypes. Analyses revealed that the O polysaccharide of Y4 LPS consists of a polymer of trisaccharide repeating units composed of D-Fuc, L-Rha, and D-GalNAc residues. An identical structure was obtained for the O polysaccharide of LPS from *A. actinomycetemcomitans* JP2, another serotype b strain. These results indicate that the serotype b antigen of *A. actinomycetemcomitans* is defined by a trisaccharide repeating unit present in the O polysaccharide of LPS.

Actinobacillus actinomycetemcomitans is a gram-negative capnophilic coccobacillus which is frequently isolated from periodontal lesions of patients with localized juvenile periodontitis (LJP) (23). Three distinct serotypes of *A. actinomycetemcomitans* were described initially, although subsequent studies have revealed the presence of two additional serotypes, as well as a number of nontypeable strains (9, 20, 24). Among LJP patients, serotype b strains of *A. actinomycetemcomitans* are recovered from subgingival plaque more frequently than are other serotypes, giving rise to speculation that serotype b strains may possess certain properties which increase their periodontopathic potential (24).

The serotype b antigen appears to be a significant target for immunoglobulin G antibodies present in sera of LJP patients colonized by *A. actinomycetemcomitans* (21, 22). Califano and coworkers observed that the immunodominant antigen of *A. actinomycetemcomitans* Y4 defines serologic specificity and exhibits characteristics of a carbohydrate (5). Amano and coworkers described the purification of a serotype b-specific polysaccharide of strain Y4 and determined the molecule to be a linear polymer composed of disaccharide units containing L-rhamnose and D-fucose (1). More recently, we isolated a high-molecular-mass polysaccharide antigen from a phenol extract of *A. actinomycetemcomitans* Y4 (22). This polysaccharide-containing antigen reacted with rabbit polyclonal antiserum to serotype b but not with antisera specific for other *A. actinomycetemcomitans* serotypes. This antigen contained substantial amounts of rhamnose and fucose but also contained constituents (3-hydroxytetradecanoic acid and L-*glycero*-D-*manno*-heptose) typically found in bacterial lipopolysaccharides (LPS). These findings prompted our conclusion that the serotype b antigen of *A. actinomycetemcomitans* is defined by the polysaccharide moiety of LPS. Page and coworkers subsequently provided evidence consistent with this hypothesis (18).

Given the immunologic significance of the serotype b antigen and its apparent localization to the polysaccharide region of LPS, we performed analyses designed to elucidate the structure of the O polysaccharide (O-PS) of *A. actinomycetemcomitans* Y4. The results indicate that the O-PS of *A. actinomycetemcomitans* serotype b consists of a repeating trisaccharide unit composed of L-Rha, D-Fuc, and D-GalNAc residues (1:1:1). This structure is unrelated to that of the O antigens of other serotypes of *A. actinomycetemcomitans.*

MATERIALS AND METHODS

Preparation of LPS and O-PS. *A. actinomycetemcomitans* Y4 (ATCC 43718, serotype b) was maintained on chocolate agar plates incubated at 37°C in humidified 5% CO_2. Broth cultures were prepared in brain heart infusion broth (Difco, Detroit, Mich.) and were incubated with constant agitation (200 rpm) at 37°C. The organisms were chilled to 4°C, killed by addition of phenol to a final concentration of 2% (wt/vol), and harvested by Sharples continuous centrifugation. The bacteria were subsequently rinsed in 0.9% NaCl and extracted with 50% aqueous phenol for 10 min at 65°C. Both the phenol and the aqueous phases were collected by centrifugation (10,000 × *g*) at 4°C and dialyzed extensively to remove phenol. The dialysates were lyophilized, dissolved in distilled H_2O, and digested sequentially with DNase, RNase, and proteinase K (Sigma Chemical Co., St. Louis, Mo.), as described previously (12). Following subsequent ultracentrifugation at 105,000 × *g* for 12 h, LPS fractions recovered as precipitated gels were dissolved in distilled H_2O and lyophilized.

Aqueous solutions (2% [wt/vol]) of LPS were hydrolyzed in 2% acetic acid at 100°C for 2 h. Precipitated lipid A was removed by low-speed centrifugation. The water-soluble fraction was lyophilized, dissolved in 0.05 M pyridinium acetate, pH 4.6, and chromatographed on a Sephadex G-50 column (3 by 92 cm) equilibrated in the same buffer. Collected fractions (10 ml) were analyzed for neutral glycose by the phenol-sulfuric acid method (6) and for 2-amino-2-deoxyglycose (7).

NMR spectroscopy. Nuclear magnetic resonance (NMR) spectra were recorded at 40°C with a Bruker AM 500 spectrometer equipped with an Aspect 3000 computer and standard Bruker software. O polysaccharides were lyophilized twice from D_2O and subsequently redissolved in D_2O to a concentration of 20 to 30 mg/ml. ^1H NMR spectra were recorded at 500 MHz by using a spectral

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width of either 2.5 or 1.25 kHz and a 90° pulse. Resolution enhancement was achieved by using a Gaussian line shape transformation. Chemical shifts are reported in parts per million relative to the value for internal acetone (2.225 ppm, 0.1%). Broad-band, proton-decoupled ^{13}C NMR spectra were obtained at 125 MHz by using a spectral width of 33 kHz, a 90° pulse, and WALTZ decoupling. Distortionless enhancement by polarization transfer spectra were obtained by using a 135° pulse and a 3.3-ms delay between pulses. Chemical shifts are reported relative to the value for internal acetone (31.07 ppm). Two-dimensional homonuclear and heteronuclear correlation experiments (COSY [2], NOESY [15], and HMQC [3]) were performed by using a spectral width of either 2.5 or 1.25 kHz, a 90° pulse, data sets ($t_1 \times t_2$) of 512 × 2,048 points, and either 32 or 48 transients.

GLC. Analytical gas-liquid chromatography (GLC) (10) was performed with a Hewlett-Packard 5890A gas chromatograph fitted with a hydrogen flame detector and equipped with a DB-17 fused silica capillary column (30 m by 0.25 mm). The following temperature programs were employed: (i) 180°C (delay, 2 min) and 2°C/min to 240°C for alditol acetate derivatives and (ii) 200°C (delay, 2 min) and 1°C/min to 240°C for acetylated *O*-methyl alditol derivatives. Retention times of glycose derivatives were recorded relative to that of 1,2,3,4,5,6-hexa-*O*-acetyl-D-glucitol (T_{GA}) or 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (T_{GM}). GLC-mass spectrometry (MS) was performed under the same conditions by using a Hewlett-Packard 5985B GLC-MS system and an ionization potential of 70 eV. Configuration of glycoses was established by capillary GLC of their derivatized 2-(*S*)-butyl glycosides (8). Retention times and corresponding mass spectra were compared with those of authentic sugar reference derivatives.

Polysaccharides were either hydrolyzed in 2 M trifluoroacetic acid in sealed tubes (5 h, 105°C) and concentrated under a nitrogen gas stream or hydrolyzed in 1 M sulfuric acid (8 h, 100°C) and then neutralized with $BaCO_3$. 2-Amino-2-deoxyglycose was removed from the hydrolysates on Rexyn 101 (H^+) resin and subsequently eluted with 1 M HCl. The polysaccharides (~1 to 2 mg) were methylated with sodium methylsulfinylmethanide and methyl iodide in dimethyl sulfoxide according to the method of Hakomori (11). Following dialysis against H_2O, the hydrolysis products were reduced with sodium borodeuteride ($NaBD_4$) and acetylated with acetic anhydride (105°C, 3 h). The resulting acetylated *O*-methyl alditol derivatives were identified by GLC-MS analysis.

Preparative paper chromatography was performed on water-washed Whatman no. 1 filter paper by using pyridine-ethyl acetate-water (2:2:5, vol/vol/vol, top layer) as the mobile phase. Specific optical rotations were determined at 20°C in 10-cm-long microtubes with a Perkin-Elmer model 243 polarimeter.

Double immunodiffusion analysis was performed in 1% (wt/vol) agarose (Calbiochem, La Jolla, Calif.) in phosphate-buffered saline, pH 7.2. LPS (1 mg/ml in distilled H_2O) recovered from the aqueous phase of a phenol-water extract of *A. actinomycetemcomitans* Y4 was transferred to the center well and reacted with rabbit antisera specific for *A. actinomycetemcomitans* serotypes a through e (provided by J. Zambon, State University of New York at Buffalo).

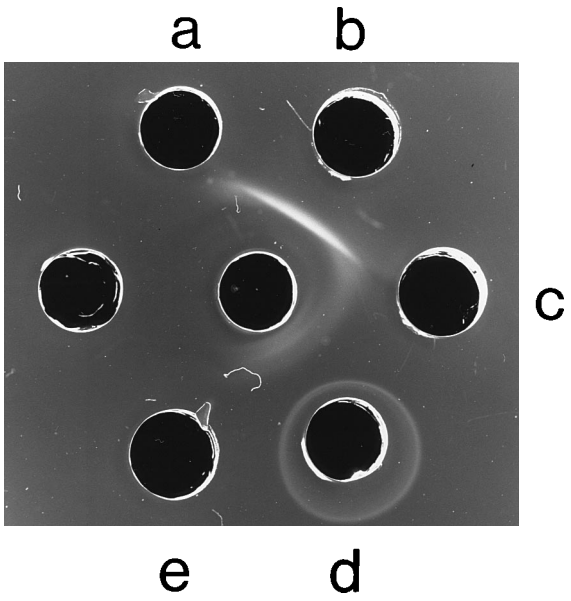


FIG. 1. Double immunodiffusion analysis of aqueous-phase LPS obtained from a phenol-water extract of *A. actinomycetemcomitans* Y4 (serotype b). Peripheral wells contained rabbit antisera against *A. actinomycetemcomitans* serotypes a to e, as indicated. The center well contained Y4 LPS (1 mg/ml in distilled H_2O).

RESULTS

Extraction of *A. actinomycetemcomitans* Y4 (53 g [wet weight]) grown in brain heart infusion broth by the hot-aqueous-phenol method yielded 294 mg of LPS from the aqueous phase and 244 mg of rough-type LPS from the phenol phase. Only the aqueous phase contained smooth-type LPS. The latter LPS fraction was tested for immunologic reactivity with rabbit antisera to *A. actinomycetemcomitans* serotypes a to e. As depicted in Fig. 1, LPS recovered from the aqueous phase was precipitated by rabbit serotype b-specific antiserum but not by rabbit antisera to the remaining serotypes.

Hydrolysis of aqueous-phase *A. actinomycetemcomitans* Y4 LPS (280 mg) in 2% acetic acid (100°C, 2 h) yielded an insoluble fraction containing lipid A (30 mg) and a water-soluble fraction which, upon chromatography on a Sephadex G-50 column, yielded an O-PS (K_{av}, 0.02, 38 mg), a core oligosaccharide (K_{av}, 0.63, 38 mg), and a monosaccharide fraction (K_{av}, 0.92, 45 mg). The O-PS had an $[\alpha]_D$ of +60° (c 0.12, water) and upon hydrolysis (1 M H_2SO_4, 8 h, 100°C), neutralization ($BaCO_3$), and preparative paper chromatographic separation yielded two neutral component sugars identified as D-Fuc (R_{Gal}, 1.38) and L-Rha (R_{Gal}, 1.65), in addition to D-GalN isolated by ion-exchange resin (Rexyn 101 [H^+]) adsorption and acid elution. GLC-MS analysis of the reduced ($NaBD_4$) and acetylated hydrolysis products showed three major peaks corresponding in retention times and mass spectra to the fully acetylated alditols of fucitol-1-d (T_{GA}, 0.63), rhamnitol-1-d (T_{GA}, 0.60), and 2-amino-2-deoxygalactitol-1-d (T_{GA}, 1.32) in a ratio of 1:1:1.

Further characterization of the component glycoses was performed with the chromatographically pure isolated hydrolysis products. The L-Rha had an $[\alpha]_D$ of +9.0° (c 0.2, water) and upon methanolysis (2% MeOH-HCl, 1 h, 100°C) gave methyl α-L-rhamnopyranoside having an $[\alpha]_D$ of −60.1° (c 0.1, water) and upon acetylation gave methyl-2,3,4-tri-*O*-acetyl-α-L-rhamnopyranoside identified by GLC-MS (program A) as a single peak (T_{GA}, 0.34). The isolated D-Fuc had an $[\alpha]_D$ of +61.0° (c 0.2, water) and upon treatment with phenylhydrazine gave D-phenylosazone having a melting point and mixture melting point of 179 to 180°C and an $[\alpha]_D$ of +58.2° (c 0.1, pyridine-ethyl alcohol) (16). The isolated D-GalN · HCl had an $[\alpha]_D$ of +91.1° (c 0.1, water), and GLC-MS analysis of its trimethylsilylated *N*-acetyl derivatives showed two major peaks corresponding in retention times and mass spectra with those of trimethylsilyl-2-acetamido-2-deoxy-3,4,6-tri-*O*-(trimethylsilyl)-α and β-D-galactopyranoside derivatives (19).

The ^1H NMR spectrum of the O-PS (Fig. 2) was consistent with the compositional analysis showing methyl proton signals from two 6-deoxyhexose residues at 1.28 and 1.33 ppm, as well as three anomeric signals of equal intensity at 4.77 ($J_{1,2} \sim 8.1$ Hz), 4.95 ($J_{1,2} \sim 1.5$ Hz), and 5.08 ($J_{1,2} \sim 1$ Hz) ppm. The ^{13}C NMR spectrum of the O-PS (Fig. 3) showed methyl carbon signals of two 6-deoxyhexoses at 16.2 and 17.3 ppm, carbon signals (underlined) from an *N*-acetyl substituent at 23.2 ($\underline{C}H_3CO$) and 175.2 ppm ($CH_3\underline{C}O$), a signal at 53.4 ppm characteristic of the C-2 of 2-acetamido-2-deoxyhexose, and three anomeric carbon signals at 98.4 ($J_{C1\text{-}H1}$, 170 Hz), 100.6 ($J_{C1\text{-}H1}$, 170 Hz), and 103.7 ($J_{C1\text{-}H1}$, 160 Hz) ppm. Anomeric signals in the ^1H and ^{13}C NMR spectra were indicative of one β and two α configurations in an O-PS composed of regular trisaccharide repeating units.

Methylation analysis of the O-PS revealed the presence of 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-D-fucitol-1-d (T_{GM}, 0.98), 1,2,3,5-tetra-*O*-acetyl-4-*O*-methyl-L-rhamnitol-1-d (T_{GM}, 1.12),

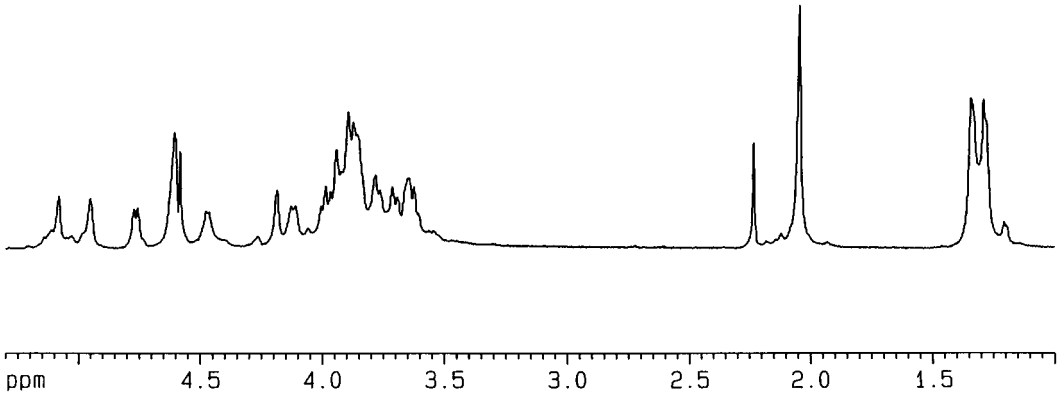
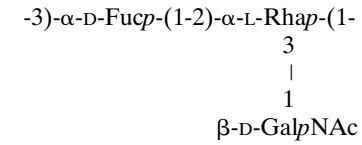


FIG. 2. ^{1}H NMR spectrum of the LPS O polysaccharide from *A. actinomycetemcomitans* Y4 (serotype b).

and 1,5-di-*O*-acetyl-2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol-1-d (T_{GM}, 2.58), indicating that the O-PS consisted of a trisaccharide repeating unit containing-3)-D-Fuc*p*-(1-, -2,3)-L-Rha*p*-(1- and nonreducing D-Gal*p*NAc end group residues.

Alkaline hydrolysis (2 N NaOH, 100°C, 2 h) of the O-PS yielded an N-deacetylated product having an $[\alpha]_D$ of +75° (c 0.3, water) which, upon periodate oxidation, reduction, mild acid hydrolysis, and subsequent chromatography on Sephadex G-50, gave a high-molecular-weight product having an $[\alpha]_D$ of +120° (c 0.10, water) composed of D-Fuc and L-Rha (1:1). The ^{1}H NMR spectrum of this residual polymer showed equal-intensity anomeric proton signals for L-Rha*p* at 5.14 ppm ($J_{1,2} \sim 1.5$ Hz) and D-Fuc*p* at 4.98 ppm ($J_{1,2} \sim 3.2$ Hz) and doublets for methyl protons of 6-deoxyaldoses at 1.30 and 1.17 ppm. An HMQC NMR experiment (3) showed the corresponding anomeric carbon signals for L-Rha*p* at 100.6 ppm ($J_{C1\text{-}H1}$ 169 Hz) and for D-Fuc*p* at 98.8 ppm ($J_{C1\text{-}H1}$ 167 Hz). Consideration of the $J_{C1\text{-}H1}$ and $J_{1,2}$ proton-coupling constants indicates that both 6-deoxyhexoses have the α configuration, whereas the D-Gal*p*NAc residues are assigned a β configuration in the O-PS.

Methylation analysis of the periodate oxidation degradation polymer product gave 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-D-fucitol-1-d (T_{GM}, 0.98) and 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl-L-rhamnitol-1-d (T_{GM}, 0.87) (1:1), indicating that a repeating disaccharide unit formed the O-PS backbone and that the nonreducing D-Gal*p*NAc residues were linked to the O-3 positions of L-Rha*p* residues. Consideration of the combined methylation and NMR analyses defines the O-PS as a regular polymer composed of trisaccharide repeating units having the following structure:



Two-dimensional COSY NMR analysis of the native O-PS allowed complete assignment of all proton signals (Table 1). The ^{13}C NMR signals (Table 1) were assigned from an HMQC experiment. Nuclear Overhauser enhancement (NOE) analysis of the O-PS showed that irradiation of H-1 of the β-D-Gal*p*NAc resulted in enhancement of its own H-2, H-3, and H-5 signals as well as that of the H-3 of L-Rha*p*. Irradiation of H-1 of D-Fuc*p* caused enhancement of its own H-2 and that of the H-2 signal of L-Rha*p*. Irradiation of H-1 of the α-L-Rha*p* residues showed a NOE to its own H-2 and the H-3 of D-Fuc*p*. The results of the two-dimensional NMR analyses confirm the above proposed linkage assignments for the trisaccharide repeating units in the O-polysaccharide structure of *A. actinomycetemcomitans* Y4 (serotype b) LPS.

The core oligosaccharide of *A. actinomycetemcomitans* Y4 LPS had an $[\alpha]_D$ of +90.0° (c 0.4, water) and was determined to be composed of D-glucose, D-galactose, D-*glycero*-D-*manno*-

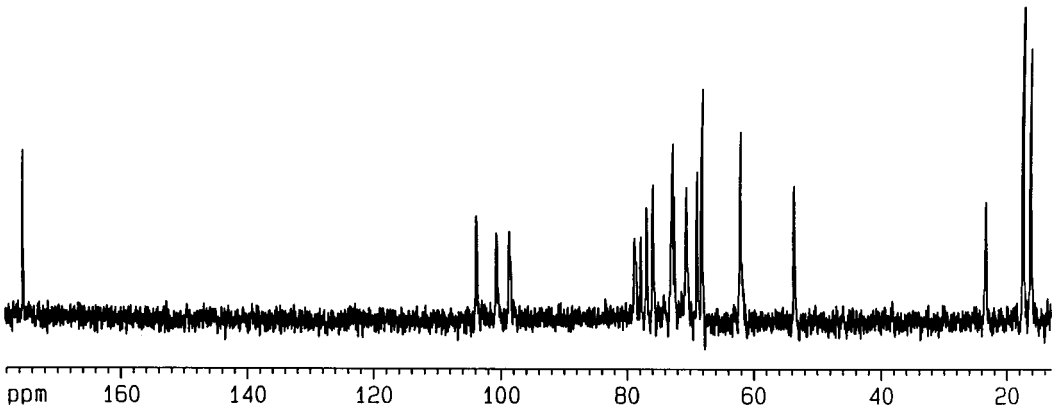


FIG. 3. ^{13}C NMR spectrum of the LPS O polysaccharide from *A. actinomycetemcomitans* Y4 (serotype b).

TABLE 1. ^{1}H and ^{13}C NMR chemical shifts for the LPS O polysaccharide of *A. actinomycetemcomitans* serotype b

Glycose residue	Chemical shift (ppm)[a]												
	H-1	H-2	H-3	H-4	H-5	H-6	H-6′	C-1	C-2	C-3	C-4	C-5	C-6
-2,3-α-L-Rha*p*-(1-	5.08 (~1)	4.19	4.12	3.62	3.87	1.33		100.6 (170)	76.8	77.6	72.9	70.5	17.3
-3)-α-D-Fuc*p*-(1-	4.95 (~1.5)	3.85	3.90	3.89	4.48	1.28		98.4 (170)	68.1	78.6	72.7	68.0	16.2
-β-D-Gal*p*NAc-(1-	4.77 (8.1)	3.98	3.71	3.94	3.64	3.77*	3.86*	103.7 (160)	53.4	72.5	68.8	75.8	61.8

[a] Coupling constants $J_{1,2}$ and $J_{C1\text{-}H1}$ (in Hertz) are indicated in parentheses. *, shift values may be interchanged.

heptose, and L-*glycero*-D-*manno*-heptose (2:1:1:2). Analysis of the lipid component revealed it to be a fatty acid-substituted disaccharide, β-D-Glc*p*N-(1-6)-D-Glc*p*N, identical to the lipid A structure of *A. actinomycetemcomitans* Y4 proposed by Masoud and coworkers (17).

DISCUSSION

Previous studies have revealed that, of the five currently recognized serotypes of *A. actinomycetemcomitans*, serotype b strains often predominate in periodontal lesions of patients with LJP. Moreover, LJP patients often exhibit markedly elevated titers of serum immunoglobulin G antibody to the serotype b antigen of *A. actinomycetemcomitans*. The prevalence of serotype b strains in LJP, as well as the apparent immunodominance of this antigen, has prompted interest in defining the nature of the serotype b antigen. Despite such interest, the precise nature of this antigen has remained an enigma.

Initial studies indicated that the serotype antigens of *A. actinomycetemcomitans* are high-molecular-weight, heat-stable carbohydrates (5, 23). Amano and coworkers (1) isolated a serotype-specific polysaccharide from an autoclaved extract of *A. actinomycetemcomitans* Y4 (serotype b) by means of ion-exchange and gel permeation chromatography. This polysaccharide was determined to be an unbranched linear polymer of a repeating disaccharide unit consisting of →3)-α-D-Fuc*p*-(1→2)-β-L-Rha*p*-(1→. The serotype b antigen was reported to contain 2.7% fatty acid, although the nature of the fatty acids was not ascertained. Those authors suggested that the serotype b antigen is distinct from the O-polysaccharide moiety of LPS.

A number of groups have observed that phenol-extracted LPS obtained from *A. actinomycetemcomitans* Y4 contains appreciable amounts of rhamnose and fucose (14, 21, 22). This prompted our initial efforts to determine the structural relationship between the serotype b antigen and the polysaccharide of LPS from *A. actinomycetemcomitans* Y4. To this end, we prepared a phenol-water extract of strain Y4 and subjected this material to gel permeation chromatography in an LPS-disaggregating buffer under conditions used to separate hydrophilic LPS and polysaccharides (13). Utilizing this approach, we obtained a high-molecular-mass polysaccharide which reacted specifically with rabbit antiserum to serotype b but not with antisera to other serotypes of *A. actinomycetemcomitans* (22). Chemical analysis revealed that the polysaccharide contained substantial amounts of rhamnose and fucose. The polysaccharide contained 2% fatty acid by weight, 24% of which was composed of 3-hydroxytetradecanoic acid, a common constituent of bacterial LPS and previously noted to be present in the lipid A moiety of *A. actinomycetemcomitans* Y4 (4, 17). L-*glycero*-D-*manno*-Heptose, another common constituent of LPS, was also present in this fraction. These LPS-associated markers and the serotype b antigen could not be physically separated by repeated chromatography on Sephacryl S-400 in LPS-disaggregating buffer, and analysis of the serotype b antigen on silver-stained sodium dodecyl sulfate-polyacrylamide gels did not reveal the presence of low-molecular-mass LPS. Hence, we concluded that the polysaccharide region of *A. actinomycetemcomitans* Y4 LPS contains the serotype b-specific antigen of this species.

The focus of the present study was to define the structure of the O polysaccharide of LPS from *A. actinomycetemcomitans* serotype b and to ascertain whether this structure or the core polysaccharide is responsible for defining serologic specificity for this organism. The O polysaccharide was found to be a polymer of trisaccharide repeating units composed of L-Rha, D-Fuc, and D-GalNAc residues. The repeating subunit structure was found to consist of -3)-α-D-Fuc*p*-(1→2)-α-L-Rha*p*-(1-, with β-D-Gal*p*NAc linked to the O-3 positions of L-Rha*p* residues. An identical structure was derived through structural analysis of the O polysaccharide of LPS from *A. actinomycetemcomitans* JP2, which is also assigned to the serotype b group. This structure is similar to the serotype b antigenic structure proposed by Amano and coworkers (1), except that rhamnose in their antigen was assigned a β-L-Rha*p* configuration and D-Gal*p*NAc was not detected as a constituent glycose.

Two lines of evidence serve to localize the serotype-specific antigen to the O polysaccharide of *A. actinomycetemcomitans*. First, the chemical compositions, specific optical rotations, and corresponding ^{1}H and ^{13}C NMR spectra of the core oligosaccharides obtained from the mild acid hydrolysates of LPS of *A. actinomycetemcomitans* serotypes a, c, d, and e were found to be identical to those of the core oligosaccharide of *A. actinomycetemcomitans* serotype b. Such data indicate that the five known *A. actinomycetemcomitans* serotypes possess a common core polysaccharide structure. Secondly, the O polysaccharides of serotypes a, c, d, and e each have unique repeating subunit structures which are distinct from that of the serotype b antigen described herein (19a).

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